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Baculovirus Expression, Purification and Evaluation of Recombinant Pneumococcal Surface Adhesin A of *Streptococcus pneumoniae*

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Key Words
Recombinant pneumococcal surface adhesin A · Baculovirus · *Staphylococcus pneumoniae*

Abstract
Pneumococcal surface adhesin A (PsaA), with a molecular mass of ~37 kD by SDS-PAGE, is a common surface protein expressed by all 90 serotypes of *Streptococcus pneumoniae*. *S. pneumoniae* serotype 6B genomic DNA was amplified to generate a DNA fragment carrying the full-length *psaA* sequence and was cloned into a baculovirus expression system. We expressed either cell-associated or cell-free nonfusion PsaA polypeptides using two insect cell lines, *Spodoptera frugiperda* (Sf9) and *Trichoplusia* ni 5B1-4 (High-Five). Recombinant PsaA (rPsaA) polypeptides were partially purified by partitioning in PBS/Triton X-114 buffers and by weakly basic ion exchange filter chromatography. Membrane-bound 'hydrophobic rPsaA' (hrPsaA) expressed by either Sf9 or High-Five cells had a molecular mass of ~38 kD by SDS-PAGE and partitioned in a Triton X-114 phase, it reacted with both rabbit polyclonal and five monoclonal anti-PsaA antibodies by dot blot or Western blot analysis. High-Five-cell-expressed 'soluble rPsaA' (srPsaA) with a molecular mass of ~37 kD by SDS-PAGE, was isolated from the serum-free culture medium and did not partition in the Triton X-114 phase; it reacted with anti-PsaA rabbit polyclonal and mouse monoclonal antibodies by ELISA and Western blot analysis. Both rPsaA polypeptide forms were immunogenic in Swiss-Webster adult female mice. In an infant mouse model of bacteremia, survival rates for mice given mouse anti-rPsaA immune serum (from mice immunized with High-Five-expressed srPsaA; 20 µl, 1:50,000 titer) 24 h before bacteremic challenge were greater than for the control group (48 h post-challenge, 20 vs. 90% survival rates) when challenged with *S. pneumoniae* serotype 6B. These results indicate that rPsaA is immunogenic and elicits protective antibody in mice similar to native protein.

Introduction

Streptococcus pneumoniae is the only cause of pneumococcal disease in young children worldwide [1–4]. It is also an important cause of sickness and death in the elder-

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ly and in persons with certain underlying medical conditions [5–7]. Pneumococcal diseases usually manifest as either bacteremia, meningitis, pneumonia, or otitis media and sinusitis. The currently licensed first-generation 23-valent pneumococcal polysaccharide (PS) vaccine provides limited protection in young children and immunocompromised individuals [8–10]. One promising approach under consideration is the use of second-generation protein-polysaccharide conjugate vaccines [11, 12], which should overcome infant nonresponsiveness, but will provide only restricted protection because of the limited number of PS-specific serotype antigens in vaccine formulations. Thus, an alternative approach is the use of third-generation vaccines composed of species-common pneumococcal protein(s), which may elicit broadly protective T-cell-dependent immunity with a long duration of protection. Several immunogenic species-common pneumococcal proteins such as pneumolysin [13, 14], pneumococcal surface protein A (PspA) [15–18], and pneumococcal surface adhesin A (PsaA) [19–25] have been identified and shown to elicit protective antibodies in mouse models.

Our laboratory identified PsaA (formerly known as 37-kD protein), a species-common protein antigen expressed by all 90 serotypes of *S. pneumoniae* [19], and characterized *psaA* as genetically conserved among pneumococcal strains tested to date [20]. It is encoded by an open reading frame of 930 bp which appears to be part of a polycistronic message [20]. PsaA is a surface-exposed polypeptide with a molecular mass of ~37 kD by SDS-PAGE and is a member of the lipoprotein receptor I antigen family [23]. It has been characterized in various ways. An antibody enzyme-linked immunosorbent assay (ELISA) using purified native PsaA as solid-phase antigen detected an increase in anti-PsaA antibody levels in convalescent-phase sera from pneumococcal disease patients with culture-confirmed *S. pneumoniae* [22]. Five monoclonal anti-PsaA antibodies [24] have been produced which react with all encapsulated strains of *S. pneumoniae*. They did not react with 55 heterologous bacterial strains causing acute lower respiratory tract diseases [19]. Active immunization with purified native PsaA produced an anti-PsaA response that protected mice against heterologous challenge with a virulent *S. pneumoniae* (strain WU2), at doses up to 45 times the LD_{50}; protective antibodies were also detected in these animals [21].

Although *S. pneumoniae* serotypes express low levels of PsaA, isolation and purification of this protein is time consuming [22]. Limited success with expression of full-length PsaA in *Escherichia coli* [22] prompted us to develop a recombinant protein expression system for PsaA to improve the yield of purified PsaA. To date, numerous eukaryotic, viral, and even prokaryotic genes have been expressed successfully using the baculovirus expression vector system (BEVS) which offers abundant expression of functional full-length foreign gene(s) under the control of the baculovirus polyhedrin promoter, *polh* [26]. We expressed full-length PsaA of *S. pneumoniae* type 6B using BEVS and analyzed the immunologic properties of the partially purified rPsaA polypeptides. We showed that the partially purified recombinant proteins elicit anti-PsaA antibodies in mice and that these anti-rPsaA antibodies reacted with the native protein and significantly improved the survival of infant mice when challenged with *S. pneumoniae* serotype 6B.

Materials and Methods

Viruses and Cells

Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant baculovirus stocks were grown and assayed at 26°C using confluent monolayers of Sf9 cells (GIBCO-BRL, Bethesda, Md., USA) in TNM-FH medium (JRH BioSciences, Lenexa, Kans., USA) supplemented with 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, Utah, USA). Suspensions of High-Five insect cells (Invitrogen Corporation, San Diego, Calif., USA) were maintained in serum-free Excell 405 medium (JRH). Gentamicin and amphotericin-B (GIBCO-BRL) were included in both media at concentrations of 50 and 0.25 μg/ml, respectively. *S. pneumoniae* strains (serotype 6B, WU2), *E. coli* and its recombinants were maintained as described previously [20].

Construction of Recombinant Transfer Vectors

S. pneumoniae type 6B genomic DNA was amplified to generate a DNA fragment corresponding to full-length *psaA* [20] by the polymerase chain reaction (PCR) using a pair of amplimers with linkers. The primer sequences were DE001:GGGGATCC**ATGAAAAAAT-TAGGTACA** [forward primer with *Bam*HI sequence (underlined) extension] and DE002:GGGAAGCTT**ATTTTGCCAATCCTTC** [reverse primer with *Hind* III sequence (underlined) extension] for PCR. The reaction mixture contained genomic DNA (10 ng), amplimers (0.2 μ*M* each), dNTPs (200 μ*M* each), 1 unit of *Taq* DNA polymerase (Boehringer Mannheim Corp., Indianapolis, Ind., USA), 10 m*M* Tris-HCl (pH 8.3), 2.5 m*M* $MgCl_2$, and 50 m*M* KCl in 100 μl of volume. Initially, a single cycle of denaturation at 95°C for 2 min was carried out followed by 35 reaction cycles consisting of annealing at 55°C for 30 s, elongation at 72°C for 2 min and denaturation at 94°C for 30 s. The amplicon (~945 bp) was purified by 'CENTRICEP' spin column (Princeton Separations, Adelphia, N.J., USA) and digested with six different restriction enzymes (*Bam*HI, *Eco*RI, *Hind* III, *Kpn*I, *Pst*I and *Xho*I) to confirm that the amplicon was identical in restriction pattern to *psaA* [20]. The full-length DNA fragment was then digested with *Bam*HI and *Hind*III and purified by agarose gel elution (Prep-A-Gene, BioRads Labs., Hercules, Calif., USA). The

gel-purified product was used in subsequent steps to generate recombinant baculovirus transfer vector.

'Bac-to-Bac' baculovirus expression system (Gibco-BRL) was selected to generate recombinant baculoviruses. This system expresses foreign gene(s) under the control of the *polh* promoter using an intermediate transfer vector (pFastBac1). The *Bam*HI/*Hind*III digested amplicon (930 bp with linkers) was inserted into *Bam*HI/*Hin*dIII cloning sites of the intermediate transfer vector by T4 DNA ligase (Boehringer Mannheim Corp.) and the ligated mixture was used to transform DH5α competent cells (GIBCO-BRL). Recombinant colonies carrying full-length *psaA* in correct orientation relative to *polh* were identified by colony hybridization using DIG labeled (Boehringer Mannheim Corp.) oligonucleotide probe complementary to the junction between the *polh* sequence and the 5′ terminus of *psaA* sequence (bold) (DE004:GCGTGCTAGCGGATCC**ATGAAAAA-ATTAGGT**). To determine whether this recombinant vector contained the original coding sequence of *psaA*, several positive clones were further analyzed by direct cycle sequencing (PRISM, Applied Biosystems, Calif., USA) of both termini using the downstream sequencing primer (TAAATATTCCGGATTATTCAT) derived from the *polh* region and the upstream sequencing primer (DE2) used for the amplification of *psaA*. One positive recombinant transfer vector (pFast37.F3) carrying the full-length *psaA* was selected to generate a second intermediate recombinant construct 'bacmid' with *psaA* under the control of *polh* by transformation of competent *E. coli* cells (DH10Bac). White recombinant 'bacmid' colonies containing *psaA* were selected by colony dot blot hybridization using a DIG-labeled oligo-probe (DE004). Several positive clones were analyzed by direct cycle sequencing of both termini and one recombinant bacmid clone (designated as pFB3) was selected for generation of recombinant baculoviruses using insect cells.

Generation of Recombinant Baculoviruses

To generate recombinant baculovirus expressing PsaA by Bac-To-Bac protocol, Sf9 cells were directly transfected with a recombinant bacmid, pFB3 in the presence of a lipofectin (GIBCO-BRL). On day 6 posttransfection, the cell supernatant was harvested and several hundred stable recombinant occlusion-negative baculovirus plaques expressing rPsaA were identified by immunodot blot assay using a rabbit polyclonal anti-PsaA antibody [19]. One of these recombinant clones (AcPsaA.F.3.11) appeared stable and highly infectious (>3 × 10^8 plaque-forming units/ml) expressing the full-length PsaA, was thus selected for subsequent studies on expression and characterization of cell-associated and cell-free rPsaA using Sf9 and High-Five cells.

Analysis of Recombinant PsaAs

To analyze expression of rPsaA by AcPsaA.F.3.11, Sf9 cells (10^4 per well) were infected with recombinant virus (5 PFU/cell) using a 24-well micro plate. At 24-hour intervals postinfection, both cells and supernatant were tested by immunodot blot assay using a rabbit polyclonal anti-PsaA antibody. Two types of insect cell lines (Sf9 and High-Five) were tested for expression of rPsaA following infection with recombinant virus. On day 3 postinfection, both the infected cell pellet and the culture supernatant were analyzed by 12% SDS-PAGE [22, 27] using the Protean II system (Bio-Rad Labs., Hercules Calif., USA). Recombinant PsaA was detected by silver staining and confirmed by Western blot analysis [19, 25] using either a rabbit polyclonal (1:5,000 dilution) or monoclonal anti-PsaA antibody (1:1,000 dilution) [24].

To isolate cell-associated rPsaA, either Sf9 monolayers (10^8 cells maintained in TNM-FH complete medium with 10% FCS) or High-Five cells (10^8 in serum-free medium) were infected at a multiplicity of infection of 5 with a plaque-purified recombinant baculovirus (AcPsaA.F.3.11). Three days postinfection, the cell pellets were extracted with 50 ml of a buffer (67 m*M* phosphate-buffered saline, pH 7.6, 2% Triton X-114) and incubated overnight at 4 °C with mixing [28]. The cell lysate was clarified by centrifugation and the clear supernatant was incubated at 37 °C for 15 min for phase separation. The viscous detergent phase (~4 ml) was separated by centrifugation and washed with 50 ml of cold 10 m*M* phosphate buffer (pH 7.6) three times. The detergent phase was resuspended in 20 ml of 10 m*M* phosphate buffer (pH 7.6) and dialyzed using a Spectra/Por membrane of molecular weight cut off (MWCO) 25,000 (Spectrum, Houston, Tex., USA) against the same buffer with three changes to remove impurities. The dialyzed detergent phase was clarified by centrifugation at 10,000 *g* for 30 min and was loaded on a weakly basic anion exchange filter column (D5; Sartorius AG, Germany) pre-equilibrated with buffer A (10 m*M* phosphate/0.1% Triton X-100, pH 7.6). The filter was then washed with 20 ml each of buffer A and buffer B (10 m*M* phosphate /0.1% Triton X-100, pH 6.5) and subsequently, eluted with 20 ml of buffer C (100 m*M* phosphate/0.1% Triton X-100, pH 6.5). All fractions were analyzed by SDS-PAGE and visualized by silver staining. Western blot analysis was also performed with an anti-PsaA rabbit antibody to detect rPsaA in these fractions.

To isolate cell-free soluble rPsaA, High-Five cells (10^8) infected with AcPsaA.F.3.11 were clarified by ultracentrifugation (rotor Type 70Ti, 50,000 rpm) for 2 h. The clear supernatant was dialyzed using Spectra/Por membrane (MWCO 25,000) against 10 m*M* phosphate (pH 7.6) with several changes and chromatographed on a D5 ion exchange filter employing the same ionic buffer sets without Triton X-100 as described above. All fractions were tested for soluble rPsaA by 12% SDS-PAGE and by Western blot using either anti-PsaA rabbit polyclonal or five monoclonal antibodies [24]. The total protein content of these fractions was determined by the BCA protein assay (Pierce, Rockford, Ill., USA) using BSA as standard.

ELISA

Immulon II flat-bottom 96-well micro plates (Dynatech Corp., Chantilly, Va., USA) were coated with either native PsaA or High-Five-expressed soluble rPsaA (50 µl, at 5 µg/ml in 10 m*M* PBS, pH 7.2) for 1 h at 4 °C. Following washes (4 × PBS-0.05% Tween-20, pH 7.2), the plates were blocked for 1 h at 37 °C using PBS-1% bovine serum albumin (BSA), pH 7.2 (BSA, EIA grade, Sigma Chemical Co., St. Louis, Mo., USA). Mouse serum samples of 50 µl (serial dilutions in PBS containing 1% BSA and 0.05% Tween-20) were added to the plates and incubated at 37 °C for 1 h. After washing, 50 µl of 1:4,000 dilution of goat anti-mouse IgG (H + L) horseradish peroxidase conjugate (Bio Rad Labs.) was added for 2 h at 37 °C. The plates were washed 4 times, incubated with substrate, 3,3′,5,5′ benzidine (TMB) (KPL, Inc., Gaithersburg, Md., USA) and the reaction was stopped by adding 50 µl of 0.18 *M* sulfuric acid. The ELISA plates were read at $OD_{450\,nm}$.

Immunization of Mice with rPsaAs

Both partially purified Sf9 cell-associated (hrPsaA) and High-Five-cell-free (srPsaA) polypeptides were used as immunogen at 2 dose levels with or without incomplete Freund's adjuvant. Adult female Swiss Webster mice were given a subcutaneous (s.c.) injection

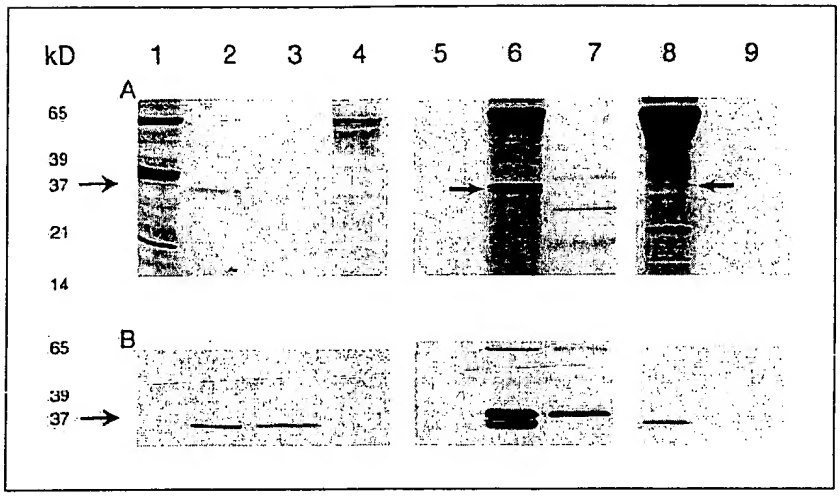

Fig. 1. SDS-PAGE (**A**) and Western blot (**B**) of rPsaA expressed in Sf9 cells 3 days postinfection. Approximately 5–10 μg of total protein was separated in 12% polyacrylamide gels. After fixing, proteins were stained with silver nitrate (**A**). Protein standards (lane 1); purified native PsaA (lanes 2 and 3); Sf9 cells infected with wild virus (lanes 4 and 5); Sf9 cells infected with AcPsaA.F.3.11 (lanes 6 and 7) and its culture supernatant (lanes 8 and 9). Lanes with odd number (3–9) represent Triton X-114-treated samples. An anti-PsaA antibody, 1E7 [24] was used for Western blot (**B**) analysis. Recombinant PsaA proteins are indicated by arrow.

with either 20 or 5 μg of rPsaAs with adjuvant at day 0 and were boosted on day 14 with an identical concentration of rPsaA without adjuvant. On day 21, animals were bled and the sera tested for anti-PsaA antibodies by dot blot assay whole cells (serotype 6B), purified native or rPsaA.

SDS/PAGE and Immunoblotting

Recombinant polypeptides were analyzed by SDS-PAGE (12%) according to the method of Laemmli [27] and proteins were visualized by silver staining [22]. For immunoblotting, proteins from 12% gels were electrophoretically transferred to nitrocellulose membranes and then probed with human sera at 1:250 dilution [22]. These serum specimens were acute and convalescent-phase sera from pneumococcal bacteremic patients and were previously obtained from a community-acquired pneumonia study in Ohio [25, 29]. Casein was used as a negative control to replace normal human serum, because majority of populations had already been exposed to *S. pneumoniae* in their childhood. The antigen-antibody complexes were detected with a goat anti-human IgG conjugated with alkaline phosphatase as secondary antibody at 1:1,000 dilution as described (Bio-Rad Labs).

Passive Protection

Infant Swiss Webster mice (10–15 days old) were subcutaneously infused with 20 μl of either control mouse sera (no immunogen) or anti-rPsaA antisera pooled from 10 mice immunized with rPsaA. Twenty-four hours after passive infusion of antibody, both immune and control mice were challenged intraperitoneally with *S. pneumoniae* type 6B ($10 \times BD_{100}$). Mice were monitored for bacteremia and survival at 48 h and at day 7; deaths were recorded at 24-hour intervals.

Active Protection

Adult Swiss Webster mice were immunized on day 0 with 5 μg of rPsaA with or without incomplete Freund's adjuvant. On day 14, a booster of 5 μg was given without adjuvant. Anti-PsaA antibody levels in these animals were determined by ELISA on day 21 postbooster, and they were challenged i.p. with *S. pneumoniae* serotype 6B strain (~700 CFU). Bacteremia was recorded at 48 h, and survival was monitored for a 7-day period; deaths were recorded at 24-hour intervals.

Results

Construction and Expression of Baculovirus Transfer Vectors, pFast37.F3 and pFB3

Both the recombinant transfer vector (pFast37.F3) and the bacmid DNA (pFB3) contained the identical full-length *psaA* (930 bp) inserted downstream to *polh* promoter as revealed by direct sequence analysis [20]. Stable recombinant baculoviruses were generated by transfection of Sf9 cells with pFB3, and the kinetics of rPsaA expression by AcPsaA.F.3.11 was studied in microtiter plates. At 24-hour postinfection intervals, both the cell lysate and culture supernatant were analyzed by immunodot assay using a rabbit polyclonal anti-PsaA antibody. On day 3 postinfection, Sf9 cells infected with AcPsaA.F.3.11 showed maximum levels of expression of rPsaA in the cell pellet as well as in the supernatant and both the Sf9 cell-associated and cell-free forms of rPsaA appeared to be stable for 10–12 days at 26°C (data not shown). After 3 days postinfection, SDS-PAGE and Western blot analysis of cell pellets and supernatant from AcPsaA.F3.11-infected with either Sf9 or High-Five cells indicated that there were two major immunoreactive bands. Their molecular masses were ~37 and ~38 kD, respectively and they co-migrated with native PsaA (fig. 1, 2; lanes 6, 7). Recombinant virus-infected cells also

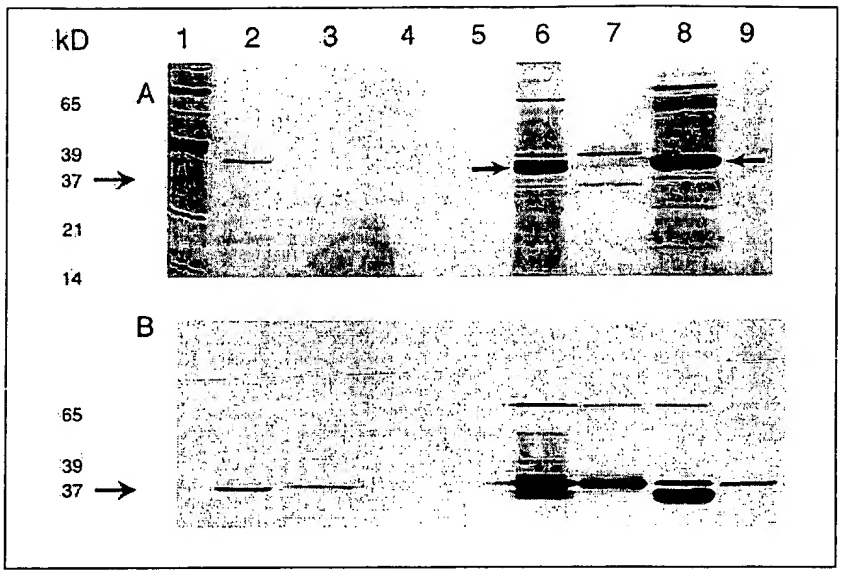

Fig. 2. SDS-PAGE (**A**) and Western blot (**B**) analysis of rPsaA expressed in High Five cells 3 days postinfection. Approximately 5–10 μg of protein was analyzed. Protein standards (lane 1); native PsaA (lanes 2 and 3); wild-virus-infected High Five cell lysate (lanes 4 and 5), AcPsaA.F.3.1-infected High Five cell lysate (lanes 6 and 7) and its culture supernatant (lanes 8 and 9). Lanes with odd number (3, 5, 7 and 9) correspond to Triton X-114-treated samples. Western blot (**B**) was performed with an anti-PsaA monoclonal antibody, 1E7 (24). The positions of PsaA polypeptides (37 kD) are shown by arrow.

consistently expressed a small amount of a third protein with a molecular mass of ~65 kD (fig. 1, 2; lanes 6, 7). This slowly migrating protein reacted also with anti-PsaA antibodies and was consistently expressed by all independent isolated recombinant baculoviruses. No other immunoreactive proteins were detected in either Sf9 cells alone or cells infected with a wild-type virus.

To investigate the differences in rPsaA expression in insect cell lines (Sf9 maintained in medium with 10% FCS and High-Five in serum-free medium), culture supernatants and Triton X-114 buffer extracts of cell pellets were analyzed by SDS-PAGE and Western blotting using an anti-PsaA mouse monoclonal antibody [19]. The majority of rPsaA associated with cell pellets appeared to have a molecular mass slightly higher than of mature PsaA (~38kD) (fig. 1; lanes 6, 7), whereas the cell-free rPsaA detected in the culture supernatants, co-migrated with the native PsaA with a molecular mass of ~37 kD (fig. 1, 2; lane 8). Interestingly, only cell-associated rPsaA (~38 kD) partitioned in the Triton X-114 detergent phase (fig. 1, 2; lane 7) thus, it was termed 'hydrophobic rPsaA' (hrPsaA) [28, 30, 31]. In contrast, the cell-free soluble rPsaA (~37 kD) did not partition in the Triton X-114 phase (fig. 1, 2; lane 9) and was designated as 'aqueous-phase soluble rPsaA' (srPsaA). Partial purification of High-Five-cell-expressed soluble rPsaA (cell-free) by weakly basic ion exchange filter (D5) chromatography appeared to remove the majority of nonspecific viral and insect cell proteins (fig. 3; lane 4).

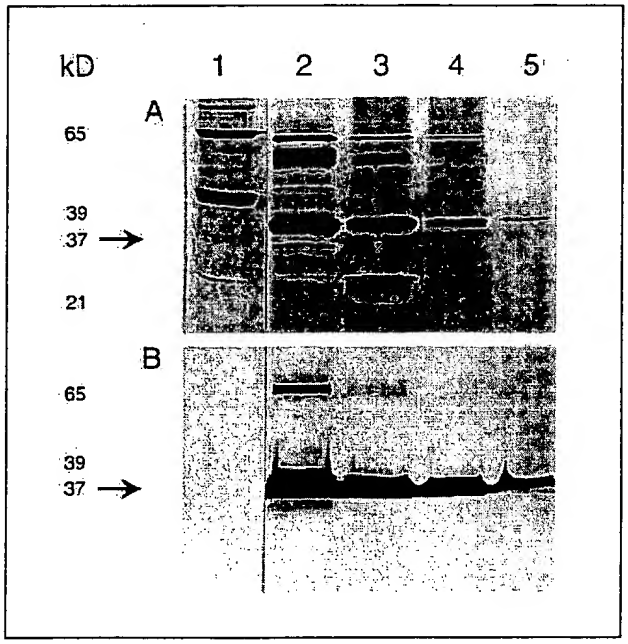

Fig. 3. SDS-PAGE analysis of partially purified soluble rPsaA expressed by High Five cells. Cell-free supernatant and partially purified protein fraction (eluted with 100 m*M* phosphate, pH 6.5) were subjected to 12% SDS-PAGE, and rPsaA was detected by silver nitrate staining (**A**) and confirmed by Western blot analysis (**B**) using an anti-PsaA monoclonal antibody, 1E7 [24]. Lane 1 = protein standards; lane 2 = culture supernatant; lane 3 = dialyzed supernatant, lane 4 = partially purified rPsaA; lane 5 = purified native PsaA as described previously [22].

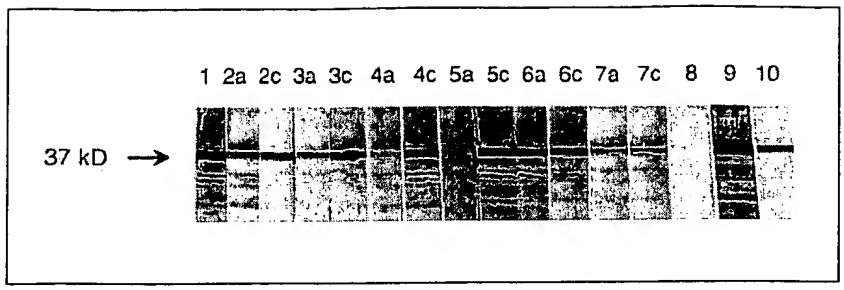

Fig. 4. Western blot analysis of human sera from patients with bacteremic and pneumococcal pneumonia using srPsaA. Purified soluble rPsaA (5 μg) was analyzed by SDS-PAGE (12%), transferred to nitrocellulose membranes and probed with human sera. Lanes 1 and 10: anti-PsaA monoclonal antibody control; lanes 8 and 9: casein and rabbit polyclonal anti-PsaA antibody controls, respectively; lanes in pair (2–7) correspond to acute- (a) and convalescent-phase (c) human sera, respectively.

An average yield of partially purified (~80%) srPsaA was estimated to be 200–250 μg/10^8 High-Five cells per 100 ml of culture supernatant as determined by the Pierce BCA protein assay using BSA as standard. However, final yield of partially purified hrPsaA was ~150 μg/l 10^8 Sf9 cells/100 ml of culture.

Authenticity of rPsaA Proteins

To test the ability of rPsaA to react with 5 monoclonal antibodies (mAbs) [24] generated against native PsaA, Western blot assays were performed. Five mAbs reacted with both hrPsaA and srPsaA polypeptides (data not shown). Additionally, analysis with High-Five-expressed srPsaA in Western blots revealed positive reactivity with six pairs of acute- and convalescent-phase sera from culture-confirmed pneumococcal pneumonia patients (fig. 4; lanes 2–7).

Passive and Active Protection

Immunized mice produced anti-rPsaA antibodies that reacted with native or recombinant protein. To determine the protective efficacy of these anti-rPsaA antibodies against pneumococcal infection, serum was collected from mice immunized with High-Five-expressed srPsaA and administered passively to infant Swiss mice. One dose of anti-rPsaA antibody (20 μl, 1:50,000 titer as determined by ELISA) administered to 20 mice by i.p. injection protected mice against challenge with 10 times the bacteremic dose (10 × BD_{100}) of serotype 6B; survival at day 2 was 20 vs. 90% and on day 7, 50 vs. 90% for the control and experimental groups, respectively. Bacteremia (48 h after challenge, >2 CFU in 10 μl blood) was reduced by as much as 60% in the animals (n = 20) that received anti-PsaA.

In an active protection model (n = 10), protection was observed at 7 days after challenge (20 vs. 60% survival); this finding is consistent with our observations with purified native PsaA [21].

Discussion

Previous studies from our laboratory have indicated that PsaA is a member of the lipoprotein receptor I antigen family and is expressed by all 90 serotypes of *S. pneumoniae* [20, 23]. It is hydrophobic by nature; the mature protein is not easily expressed in an *E. coli* system using its signal peptide [20]. Recently, numerous prokaryotic genes have been successfully expressed under the control of their own leader peptides using BEVS [34–38]. This insect eukaryotic expression system has several advantages: (1) no bacterial lipopolysaccharides, (2) allows proper translocation of recombinant protein, (3) overexpression of functional gene, (4) no host cell cross-reactivity, (5) capable of expression of multiple genes, and (6) less sensitive to proteolysis.

In this study, we explored the use of BEVS to produce high quantities of rPsaA and demonstrated the expression of full-length PsaA polypeptides under the control of their leader peptide in insect cells. Using *E. coli* 'bacmid' DNA technology, we generated stable recombinant baculoviruses expressing full-length PsaA with a high degree of infectivity (>10^8 infectious plaques/ml). Two insect cell lines, Sf9 and High-Five, were tested to compare the levels and quality of expression. In both cases, two major forms of nonfusion rPsaA polypeptides (~37 and ~38 kD), along with small amounts of a third immunoreactive protein with a molecular mass of ~65 kD were expressed. We also noticed that High-Five cells, which usually confer higher levels of expression of recombinant proteins [32, 33] even in serum-free medium, produced more cell-free soluble '37 kD' (>50% of total proteins) compared with Sf9 cells, in which the major product was mostly '38 kD' along with minor amounts of '37 kD' rPsaA polypeptides.

Recombinant polypeptides appear to be relatively stable and closely resemble native PsaA. The higher levels of expression in serum-free medium were an advantage for

the purification of cell-free rPsaA (37 kD) since the enrichment step (Triton X-114 phase separation) is not useful with proteins that do not partition in the detergent phase. After dialysis and weakly basic (D5) filter ion exchange chromatography, the final purified preparation consisted of mostly soluble rPsaA (approximately 80% pure). In the case of cell-associated recombinant protein, we chose to isolate Sf9 or High-Five cell-associated 'hrPsaA' by solubilization of cell lysate with 2% Triton X-114 buffer and removal of Triton X-114 and cellular lipoproteins by D5 weakly basic ion exchange chromatography using buffers containing Triton X-100. These preparations of hrPsaA appear to be relatively stable in these buffers (approximately >75% pure) based on densitometric analysis of silver-stained gels of SDS-PAGE (data not shown). Among the nonionic detergent buffers tested (data not shown), Triton X-114 allowed the maximum solubilization and phase separation of rPsaA at elevated temperature [31]. We estimate our total yield of purified protein to be approximately 3.0 mg/l culture. This is ~3- to 4-fold higher than that reported previously [22] for the yield of native protein using the isoelectric focusing method of purification.

At present, we know very little about the hydrophobic PsaA and its recombinant forms in terms of antigenic epitopes, the nature of the hydrophobicity, and its potential roles in eliciting protective anti-PsaA antibodies. Studies are ongoing in our laboratory to resolve these issues. Both insect cell lines express three molecular mass (37, 38, 65 kD) forms of PsaA. The higher molecular mass of the cell-associated 'hrPsaA' (38 kD) might be attributed to incomplete posttranslational acylation in insect cells [26] or retention of the hydrophobic signal sequence at the N-terminus, which allows formation of hydrophobic protein complexes with cellular membrane proteins. Similar molecular mass differences have been noted in expression of native PsaA by *S. pneumoniae* strains [20]. The lower molecular mass cell-free 'srPsaA', which co-migrates with native PsaA during SDS-PAGE and partitions with the Triton-X114 aqueous phase during separation, is most likely a hydrophilic polypetide. Therefore, in the case of High-Five cells, signal peptidase II might play a minimal role during posttranslational modifications and thus allow production of mostly cell-free soluble rPsaA [33]. The reason for expression of high-molecular-weight protein (~65 kD) was not studied in detail, but its expression by both insect cells might be explained by intracellular association of rPsaA with other host lipoproteins [26] or self-dimerization, as reported earlier [26].

The immunogenicity data presented here provide evidence that both forms of the polypeptide are immunogenic. Western blot analysis with anti-PsaA antibodies (five monoclonal and one rabbit polyclonal) showed reactivity with both hydrophobic and soluble rPsaA. Both polypeptides were immunoreactive with anti-PsaA antibodies found in acute- and convalescent-phase human serum from patients with culture-confirmed pneumococcal infections. Additionally, limited studies of mice immunized with either recombinant polypeptide demonstrated high levels of anti-PsaA antibodies in their sera; and passive protection studies with the antibodies elicited by immunizing mice with srPsaA showed that they afforded protection to mice from lethal challenge with virulent *S. pneumoniae* serotype 6B.

Recently, several bacterial membrane-associated antigens [34–38] were successfully expressed using BEVS and were evaluated as potential vaccine candidates or as alternative carriers for capsular polysaccharide vaccines. Our studies suggest that the baculoviruses are good vectors for efficient expression of immunogenic, full-length, cell associated and cell-free bacterial antigens in large amounts. Further studies of the molecular and immunogenic characteristics of rPsaA will enable evaluation of these protein recombinant polypeptides as potential third-generation vaccine candidates, possibly alone or more likely in combination with other pneumococcal common proteins. Successful development of a common protein vaccine will result in a broad-spectrum pneumococcal vaccine that is T-cell dependent, offers a wide range of protection, and elicits memory responses.

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